Determination of Estrogenic Activity in Beer by Biological and Chemical Means

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It has been suspected that beer drinking may change the hormonal status of men caused by phytoestrogens. Five different Austrian lager beers have been investigated for estrogenic activity by a yeast two-plasmid system harboring the human estrogen receptor α, after concentration by solid phase extraction. The beer concentrate was further fractionated by reversed phase HPLC, and then the fractions were characterized by the biological assay and GC-MS. The most potent fraction did not contain a known phytoestrogen. The total activity corresponded to an average of 43 ng of 17*β*-estradiol/L of beer. It was concluded that the human health hazard of beer drinking originating from compounds activity on the estrogen receptor α is negligible.

Keywords: *Phytoestrogens; beer; yeast; isoflavonoids*

INTRODUCTION

Estrogenic activity in beer and hop extracts has been already described in the early 1950s (*1*). Phytoestrogens have been identified and made responsible for the estrogenic activity (*2*−*4*). These compounds have been classified into five groups: flavones, flavanones, isoflavones, coumestans, and dihydrochalcones (*5*−*7*). Isoflavonoids have been identified in Czech beer by Lapcik et al. (*8*). The low potency of these compounds cannot account for the entire estrogenic activity in beer. Milligan et al. (*9*) identified a potent phytoestrogen in hops (*Humulus lupulus* L.) and beer, 8-prenylnaringenin, which has an activity greater than that of other established plant estrogens. The presence of 8-prenylnaringenin in hops may provide an explanation for the accounts of menstrual disturbances in female hop workers. This phytoestrogen can be also detected in beer, but the levels are low. The health effects of estrogenic active compounds in beer and other alcoholic beverages are still controversial (*10*−*13*). Couwenbergs (*10*) reported significant hormone changes in males consuming wine and beer. Other reports deny any health impact caused by estrogenic activity present in beer (*12*, *13*). Another concern is the food−drug interaction, which has been raised for grapefruit juice. Grapefruits contain different bioflavanoids, for example, naringenin, which inhibit various enzymatic processes. For this reason there is a ban on the combination of various drugs with grapefruit juice (*14*). A similar effect could be possible for estrogenic activity in beer. The increasing production and use of xenoestrogens in the environment raise the question of the consequences on nutrition, humans, and wildlife (*15*, *16*). It is very important to know the actual concentration in food to predict a reasonable risk assessment. The effects can be recorded easily with in vitro tests, which simulate the biological functions of endocrine disrupters.

With regard to this background, beer has been analyzed with a two-plasmid yeast test for its capacity to transactivate the human estrogen receptor (hER) α. For the identification and characterization of estrogen-active substances, all active samples have been further investigated by chemical analysis. The following substances were chosen for determination: estrogens 17*β*-estradiol, 17α-estradiol, 17α-ethinylestradiol, estriol, estrone, and mestranol; phytoestrogens biochanin A, coumestrol, daidzein, diosmetin, equol, formononetin, genistein, hesperetin, naringenin, phloretin, and tangeretin.

MATERIALS AND METHODS

Chemicals and Reference Compounds. All solvents were purchased from J. T. Baker (Phillipsburg, NJ). The standard substances daidzein, biochanin A, hesperetin, naringenin, phloretin, equol, 17*β*-estradiol, 17α-estradiol, 17α-ethinylestradiol, estriol, mestranol, and estrone were purchased from Sigma-Aldrich-Fluka (Vienna, Austria). The deuterated standards estron-d_4 and 17*β*-estradiol-d_5 were obtained from the University Hospital for Children and Youth Health Care in Graz (Austria). Coumestrol, genistein, formononetin, and tangeretin were purchased from Indofine Chemical Co. (Sommerville, NJ). All compounds for derivatization to enable gas chromatographic analysis were purchased from Sigma-Aldrich-Fluka.

Standard Solutions. Stock solutions of all reference compounds were made in a concentration of 1 mg/mL in methanol except coumestrol, which was dissolved in dimethyl sulfoxide (DMSO). Mixtures with 10 ng/*μ*L in methanol have been made for working solutions. Experiments were first made with these mixtures endowed with distilled water. For quantification by means of GC-MS the two deuterated substances estrone and estriol were used.

High-Performance Liquid Chromatography (HPLC) with Diode Array Detection (DAD). An HPLC system from

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Hewlett-Packard Series 1050 (Vienna, Austria) connected with an HP diode array detector 1040 M was used. A LiChrospher 60RP select B Vertex column from Knauer Säulentechnik (Berlin, Germany) was used for separation. The dimensions were 250 mm × 4 mm and 5 μm particle size. A gradient from 90% H_2O and 10% acetonitrile (ACN) (v/v) to 5% H_2O and 95% ACN (v/v) for 35 min was generated. The column was operated at a flow rate of 1 mL/min (*17*, *18*). For identification of all substances UV spectra and retention times were used. The settings of the eight DAD channels were made after the determination of the wavelength of the absorption maxima from all spectra. Fractions have been collected with the fraction collector FRAC-100 (Amersham Pharmacia Biotech, Uppsala, Sweden) and evaporated on a UNIVAP UVC 150 (UniEquip, Martinsried, Germany). The residues were derivatizised for GC-MS sampling as described below and diluted in DMSO for the yeast cell test.

Derivatization. The standard substances were derivatized for the GC-MS measurements. First, the concentrated samples were vaporized to dryness with nitrogen. Then a volume of 100 μL of the derivatization mixture containing bis(trimethylsilyl)trifluoracetamide with 1% trimethylsilane and 2% trimethylsilylimidazole was added and kept for reaction at 60 °C for 30 min (*19*, *20*).

Gas Chromatography—Mass Spectrometry (GC-MS). For the GC-MS analysis a gas chromatograph from Hewlett-Packard HP 5890 Series II was connected to a mass spectrometer HP 5971A. A Chrompack WCOT (Resteck, Vienna, Austria) fused silica 30 m × 0.25 mm × 0.25 μm CP-SIL 8CB low-bleed/MS column was used. As carrier gas helium was applied at a flow rate of 1 mL/min. The injection volume was 1 μL, and the injector and detector temperatures were 270 and 280 °C, respectively (*21*, *22*). The following temperature program has been developed: start temperature, 110 °C for 1 min, ramped at 15 °C/min to 230 °C, second ramp at 2 °C/min to a temperature of 260 °C, and finally heating at 15 °C/min to 280 °C, holding at this value for 5 min.

The first measurement was made in the scan mode for the determination of retention times and characteristic mass spectra at 70 eV. Then a selected ion monitoring (SIM) program was developed, and the samples were analyzed with this method.

Yeast Strain and Growth Conditions. Yeast strain 188R1 (*Saccharomyces cerevisiae*) transformed as a two-plasmid system with an hER α expression plasmid (YEpE12; CUP1 promotor) and a corresponding reporter plasmid (YRpE2) containing two copies of the vitellogenin ERE and the iso-1-cytochrome *c* (CYC1) promoter in a lacZ fusion vector was used (*23*−*25*). The auxotrophy marker for the expression plasmid was tryptophan (Trp) and for the reporter plasmid uracil (Ura). For every test run a new overnight culture was taken (30 °C, 200 rpm). Gold medium without Trp/Ura was used for yeast cultivation.

Pretreatment of Beer Samples. For removal of CO_2, 500 mL of beer was slowly filtered through a folded filter (Schleicher und Schüll, Düren, Germany) to avoid foam formation. Then the sample was brought to pH 3.5 by titration with concentrated H_2SO_4. To 100 mL of solution was added 5 mL of acetone, and the mixture was further concentrated 100-fold by solid phase extraction on a 3 mL Bakerbond spe* ocatdecyl (C_{18}) disposable extraction column (J. T. Baker). Columns were dried with nitrogen under vacuum conditions. The adsorbed solutes were eluted with 6 mL of pure acetone. All liquids were flushed through the solid phase extraction column by connecting it to a water-jet vacuum pump. The effluent was dried in a vacuum with rotavapor. The dry matter was redissolved in 1 mL of DMSO. These samples were subjected to further analysis.

Design of the Experiments. Four different brands of beer (Gösser Märzen, Gösser spezial, Ottakringer Goldfassl spezial, and Stiegl Goldbräu) were investigated for their estrogenic activity.

Pretreated samples (10, 30, and 50 μL) were added to the yeast culture medium containing the cells. The transactivation test was performed in 5 mL cultures (in 50 mL jars). Therefore, the overnight culture was diluted to $OD_{600} = 0.5$. hER expression was induced by the addition of 10 μmol/L $CuSO_4$. For all preparations the same volume of DMSO was added to the yeast cultures. After induction of 4 h at 30 °C, the yeast cells were extracted. DMSO alone was used as blank. A calibration curve was made with 17β-estradiol within every test run.

Preparation of Yeast Extracts. For transactivation assays yeast solutions were centrifuged (1000*g*, 5 min), the pellets were resuspended in 1 mL of lacZ buffer (100 mmol/L sodium phosphate buffer, pH 7.0, containing 10 mmol/L KCl, 1 mmol/L $MgSO_4$, and 50 mmol/L β-mercaptoethanol), centrifuged (8944*g*, 5 min, 4 °C), transferred to test tubes, and accordingly extracted with 100 μL of lacZ buffer. Disintegration of yeast cells was made by vortexing with glass beads (four times for 30 s with a 15 s rest on ice between intervals). After centrifugation (8944*g*, 10 min, 4 °C), β-galactosidase assay and protein assay were performed.

β-Galactosidase Assay and Protein Determination. The amount of expressed β-galactosidase was determined basically as described by Lyttle et al. (*23*). Briefly, 2 μL of the lysed cell suspension were pipetted into the wells of a microtiter plate and replenished with 250 μL of chromogenic substrate (4 mg of ONPG/mL of lacZ buffer). After the plate had been incubated at 37 °C, the color reaction was stopped by adding 100 μL of 1 mol/L Na_2CO_3. The resulting absorption was measured at 405 nm with a SLT EAR 400 AT plate reader (SLT, Salzburg, Austria). The quantification of total protein amount was determined with the Bio-Rad protein assay reagent. A BSA standard curve was created within every protein assay. The specific enzyme activity was expressed in Miller units (*26*), which take the amount of total protein into account. They are defined as follows, where Δ*t* is the incubation time at 37 °C in minutes:

$$\text{Miller unit} = \frac{OD_{405}}{\mu\text{g of protein mL}^{-1}} \times \frac{1}{\Delta t} \times \frac{\text{sample vol of protein assay}}{\text{sample vol of } \beta\text{-gal assay}} \times 1000 \quad (1)$$

Each determination was carried out in duplicates.

Curve Fitting. Data derived from transactivation assays were fitted using a logistic dose−response equation to approximate the concentration-dependent effect of a ligand on transactivation. Table Curve 2D software (Jandel Scientific) was used for calculation. The function is described as

$$Y = a + \frac{b}{1 + (x/c)^d} \quad (2)$$

Parameter *a* equals the baseline, and *b* is the plateau of the curve designated as the ligand efficiency. Parameter *c* gives the transition center and equals the ligand potency, which is the concentration that causes 50% efficiency (*25*, *27*, *28*).

RESULTS

Four different brands of lager beer were investigated for their capacity to transactivate the human estrogen receptor α. A minimal receptor-mediated transactivation system was reconstituted in yeast (see Materials and Methods). Activation of the estrogen receptor α yielded to a switch on of the reporter gene, which is under control of a vitellogenin hormone response element. For the purpose of transactivation measurement, the beer samples were degassed and concentrated by solid phase extraction on a C-18 column. The eluate was dried and reconstituted in DMSO. From previous experiments with synthetic mixtures, we knew that phytoestrogens and estrogens are highly soluble in this solvent and that it is compatible with the yeast test system. Up to 1% (v/v) DMSO in the yeast culture medium does not harm

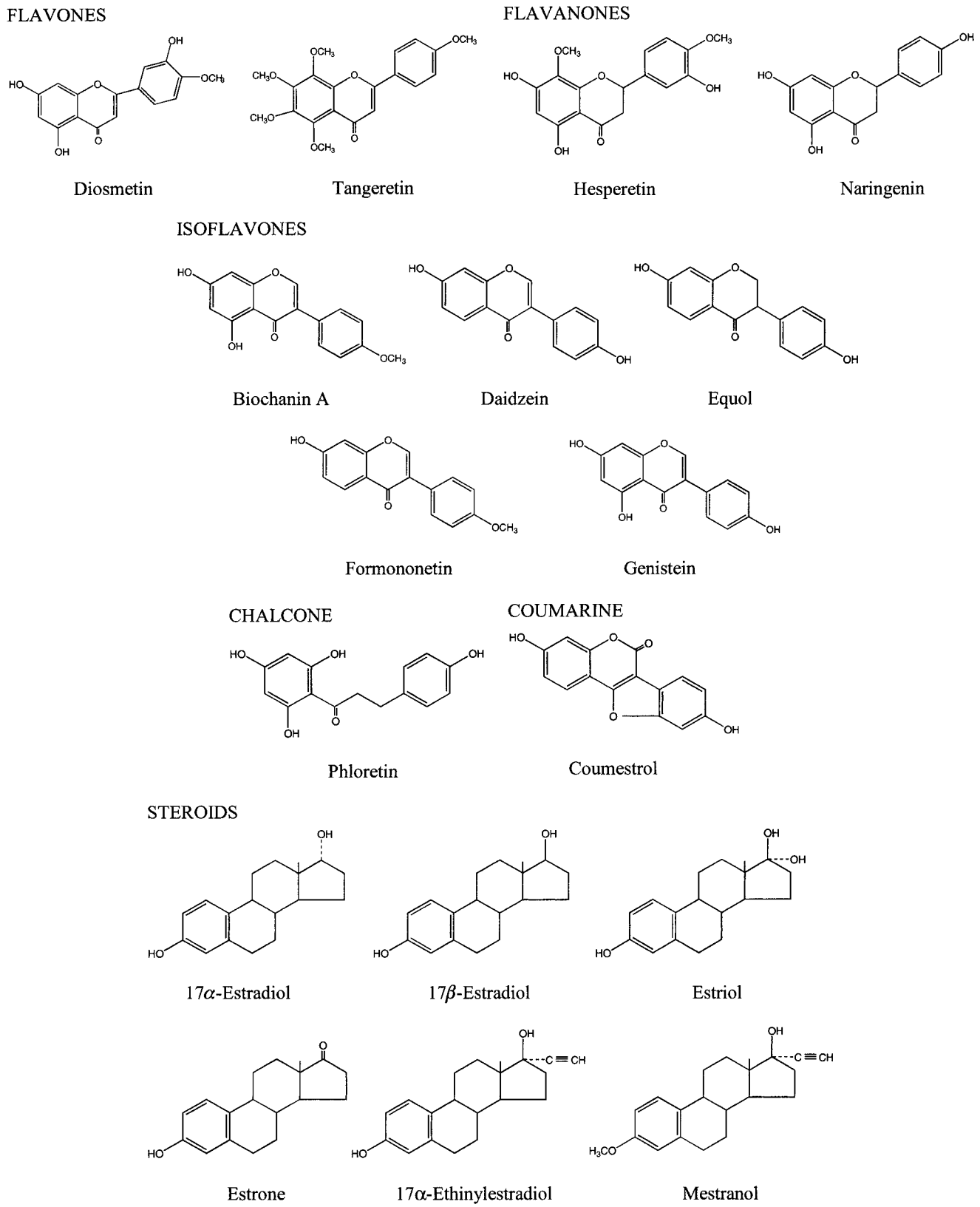

Figure 1. Chemical structure of selected phytoestrogens and natural and synthetic estrogens.

the culture. Then the estrogenic activity of these samples has been assessed by an in vitro assay as described under Materials and Methods and fractionated by reversed phase HPLC. In parallel to measuring estrogenic activity in beer, we have determined the limit of detection with pure reference compounds. The structures of these compounds are shown in Figure 1. For the transactivation assay the compounds were also diluted in DMSO and transactivational capacity was determined. The obtained data pairs were fitted by eq 2. The dose−response curves of four selected phytoestrogens are shown in Figure 2. Potency and efficiency of all tested compounds were calculated from the fit. The efficiency of the compounds was related to the natural 17β-estradiol and expressed as percentage of 17β-estradiol efficiency (Table 1). For those com-

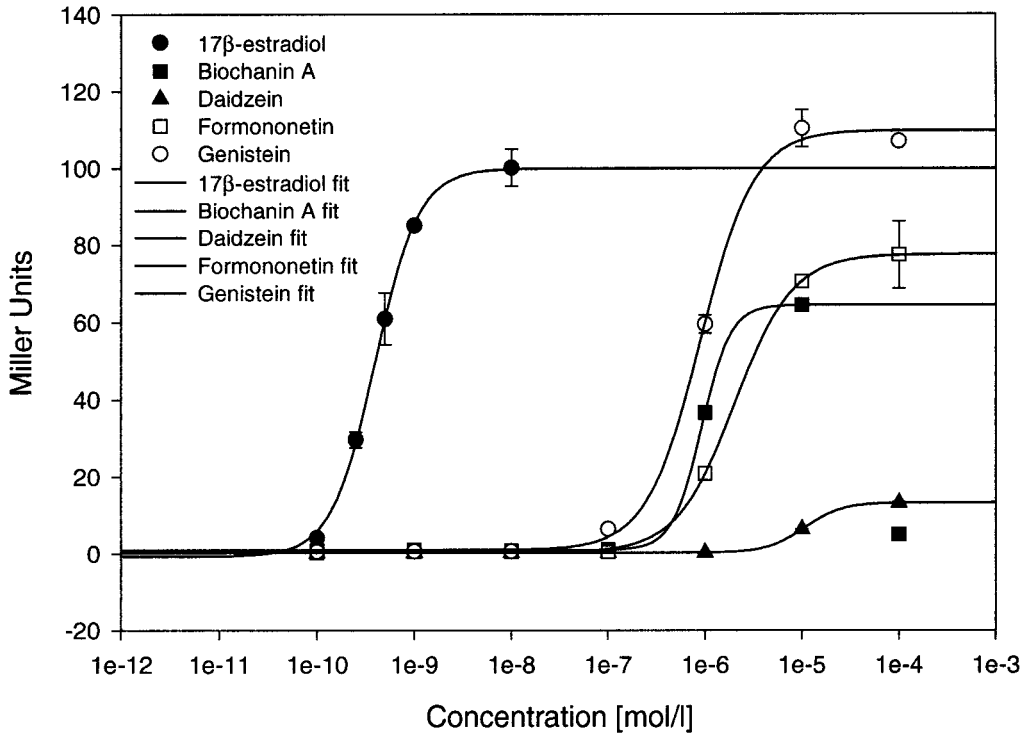

Figure 2. Dose−response curve of the four main phytoestrogens. Experimental data were approximated by logistic dose−response function.

Table 1. Determination of the Activity of the Standard Substances in Relation to 17β-Estradiol

substance	group	efficiency (%)	potency (mol/L)	detection limit (mol/L)	tested concn range (mol/l)
biochanin A	isoflavone	67	9.9×10^{-07}	1×10^{-11}	10^{-04}–10^{-10}
coumestrol	coumarin	108	5.0×10^{-08}	1×10^{-08}	10^{-06}–10^{-12}
daidzein	isoflavone	14	1.0×10^{-05}	1×10^{-05}	10^{-04}–10^{-10}
diosmetin	flavone	<1[a]	1×10^{-05}[b]	1×10^{-04}	10^{-04}–10^{-10}
equol	isoflavone	119	5.6×10^{-07}	1×10^{-07}	10^{-04}–10^{-10}
formononetin	isoflavone	76	2.0×10^{-06}	1×10^{-06}	10^{-04}–10^{-10}
genistein	isoflavone	107	9.0×10^{-07}	1×10^{-07}	10^{-04}–10^{-10}
hesperetin	flavanone	2[a]	1×10^{-05}[b]	1×10^{-04}	10^{-04}–10^{-10}
naringenin	flavanone	80[a]	3.8×10^{-05}	1×10^{-05}	10^{-04}–10^{-10}
phloretin	chalcone	50[a]	7.7×10^{-05}	1×10^{-05}	10^{-04}–10^{-10}
tangeretin	flavone	2[a]	1×10^{-04}[b]	1×10^{-04}	10^{-04}–10^{-10}
17α-estradiol	steroid	108	3.6×10^{-09}	1×10^{-10}	10^{-06}–10^{-12}
17β-estradiol	steroid	100	4.1×10^{-10}	1×10^{-10}	10^{-08}–10^{-10}
ethinylestradiol	steroid	139	1.1×10^{-09}	1×10^{-10}	10^{-08}–10^{-12}
estrone	steroid	109	3.7×10^{-09}	1×10^{-10}	10^{-06}–10^{-12}
estriol	steroid	113	8.0×10^{-08}	1×10^{-07}	10^{-04}–10^{-10}
mestranol	steroid	124	7.0×10^{-09}	1×10^{-11}	10^{-06}–10^{-12}

[a] Saturation could not be achieved within the tested concentration range. [b] Significant transactivation recognizable starting at this concentration.

pounds that did not reach saturation, the efficiency was calculated with the highest concentration tested. The limit of detection was defined as the transactivational efficiency above twice the efficiency measured with the blank.

The transactivational capacities of the four beers are given in Table 2. In three brands a low transactivational potency was detected; in the fourth brand a transactivational activity could not be detected with our system. The potency was related to that 17β-estradiol concentration producing the same activation. The test did not reach saturation; therefore, efficiency data were not provided (Figure 3).

For the determination of phytoestrogens by HPLC and GC-MS, pure reference compounds were tested. Furthermore, beer concentrate was spiked with dilutions of phytoestrogens and then analyzed by HPLC.

Table 2. Equivalent Transactivation Potential of Different Brands of Beer

brand of beer	equivalent transactivation potential (mol of 17β-estradiol/L of beer)
Gösser Märzen	8.57×10^{-11}
Gösser Spezial	not detectable[a]
Ottakringer Goldfassl spezial	1.5×10^{-10}
Stiegl Goldbräu	9.9×10^{-11}

[a] Less than 5×10^{-11} (mol of 17β-estradiol/L of beer).

The limit of detection of the various phytoestrogens was 10 μg/L. The retention data are summarized in Table 3. When the same samples were analyzed after derivatization by GC-MS, the detection limit was substantially lower (Table 4). 17β-Estradiol-d_5 and estrone-d_4 denote deuterated standards for recovery and as internal standard. Especially the estrogens had a good recovery

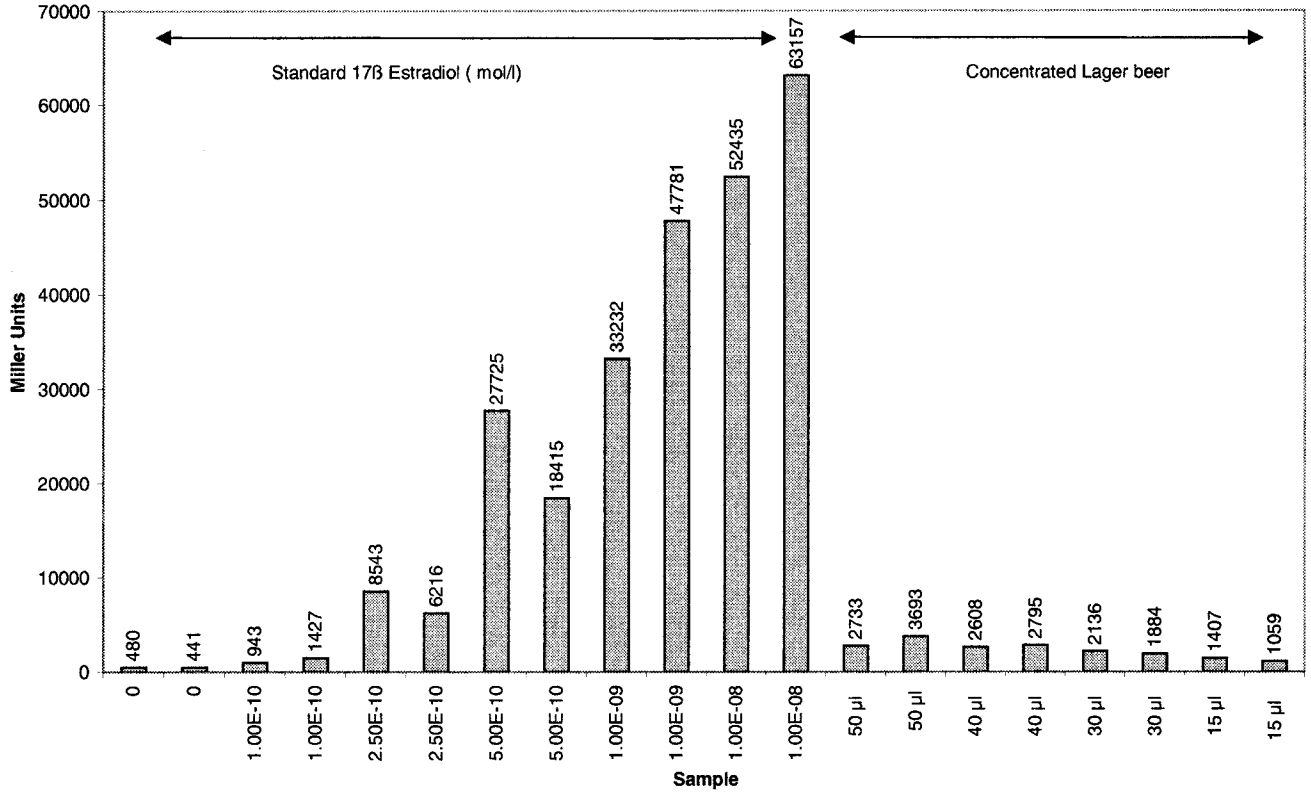

Figure 3. Estrogenic activity of concentrated beer measured by the in vitro assay using the two plasmid system.

Table 3. HPLC Retention Times of Phytoestrogens and Estrogens

substance	retention time (min)	substance	retention time (min)
daidzein	13.6	formononetin	17.9
estriol	13.9	17α-estradiol	19.5
phloretin	16.2	17β-estradiol	19.5
diosmetin	15.9	17α-ethinylestradiol	20.0
naringenin	16.4	estrone	20.2
equol	16.6	biochanin A	20.3
genistein	16.7	tangeretin	21.3
hesperetin	16.8	mestranol	24.8
coumestrol	16.9		

[a] The HPLC method has a detection limit of 1 μg/100 mL of beer for all substances. The wavelength traces for the DAD were programmed in different channels: channel A, 210 nm; channel B, 250 nm; channel C, 260 nm; channel D, 290 nm; channel E, 343 nm; channel F, 280 nm; channel G, 310 nm.

Table 4. Selected Ions, Limits of Detection, and Retention Times of Standard Substances for GC-MS Analysis[a]

substance	selected ions (m/z)[b]	retention time (min)	detection limit (ng/L)
biochanin A	413, 370, 340	21.45	1000
coumestrol	412, 397	25.63	200
daidzein	398, 383, 184	22.25	200
equol	386, 192, 177	15.54	5
formononetin	340, 325, 132	20.42	300
genistein	414, 399, 192	22.70	900
hesperetin	503, 222, 192	21.95	500
naringenin	473, 296, 488, 474	21.15	300
phloretin	369, 342, 192, 179	17.82	80
17α-estradiol	416, 285, 129	16.90	10
17β-estradiol	416, 285, 129	17.70	10
17α-ethinylestradiol	421, 287, 131	19.95	10
17β-estradiol-d_5	425, 285, 231	17.68	10
estriol	504, 345, 311	21.98	10
estrone	342, 257	17.15	10
estrone-d_4	346, 261, 220	17.12	10
mestranol	367, 115	19.00	10

[a] Diosmetin and tangeretin cannot be analyzed by GC-MS. Tangeretin cannot be derivatizised. Both substances are too unstable for GC analysis. [b] $z = 1$.

rate of ~90%. The phytoestrogens showed a lower recovery, in the range of 80%. The selected ions and retention times for the determination of phytoestrogens are also given in Table 4.

Two concentrates of beer with the highest estrogenic activity (Ottakringer Goldfassl spezial, see Table 2) have been selected for preparative separation by reversed phase HPLC analysis. The beer concentrates corresponding to 25 mL of lager beer were injected, and 1 mL fractions were collected. The fractions were vacuum-dried, diluted in DMSO (reaching injection volume), and further analyzed for estrogenic activity by the in vitro assay (Figure 4). For both concentrates a transactivational activity only in fraction 21 corresponding to equivalent activities of 1.5×10^{-10} mol of 17β-estradiol/L of beer (41 ng of 17β-estradiol/L of beer) and 1.67×10^{-10} mol of 17β-estradiol/L of beer (46 ng of 17β-estradiol/L of beer), respectively, could be found. The beer concentrates correspond to 1.5×10^{-10} and 1.63×10^{-10} mol of 17β-estradiol/L of beer, respectively. As there was one fraction showing significant estrogenic activity, this fraction did not contain any of the tested reference compounds. The corresponding chromatograms of preparative HPLC separation are shown in Figure 5. From the spectrum and the GC-MS analysis, the presence of 8-prenylnaringenin recently described by Milligan et al. (*9*) could be excluded. They reported an extremely low content of this compound in beer and also stated that their yeast assay did not detect 8-prenylnaringenin. The weak estrogenic activity in our samples must be caused by an unknown agonist. Unspecific activation can be excluded because the yeast culture is standardized for growth rate and the reporter gene expression is standardized for total cell mass.

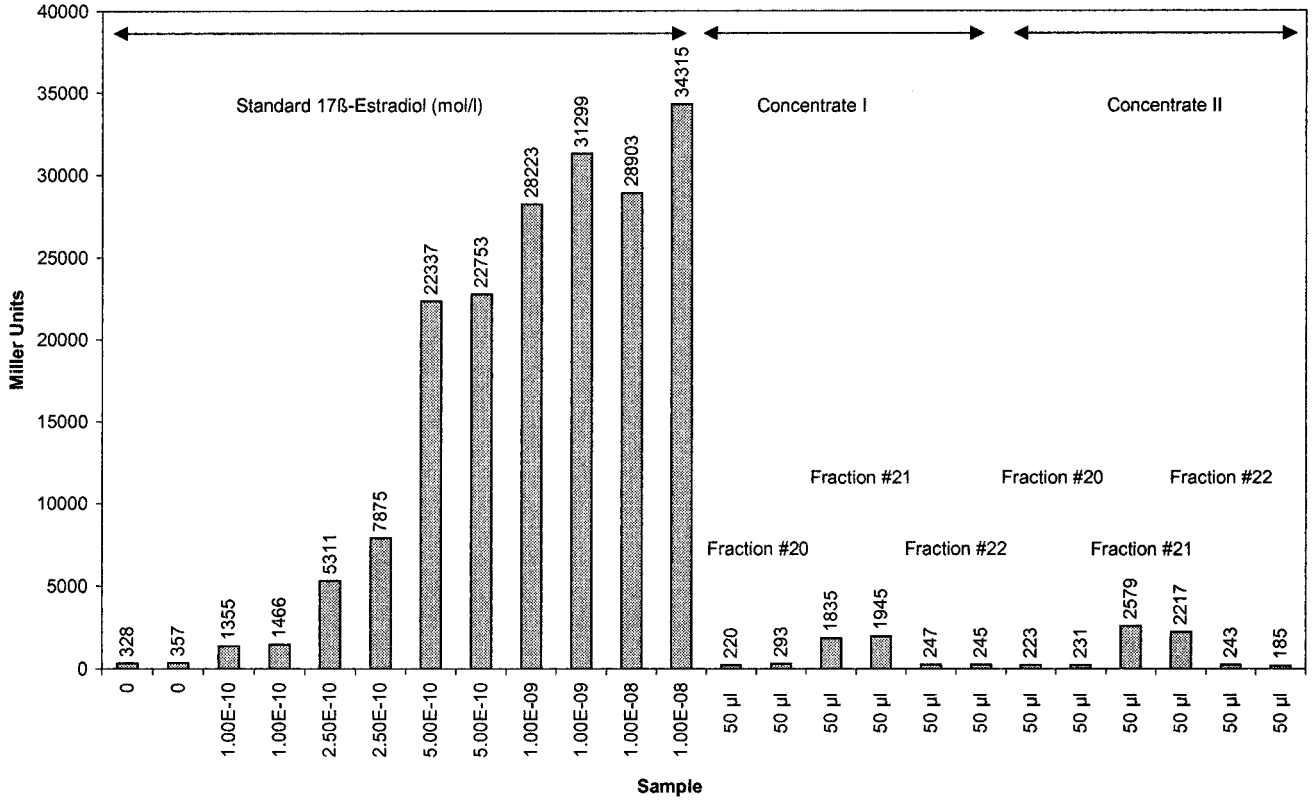

Figure 4. Estrogenic activity of fractions of beer concentrates showing the highest activity.

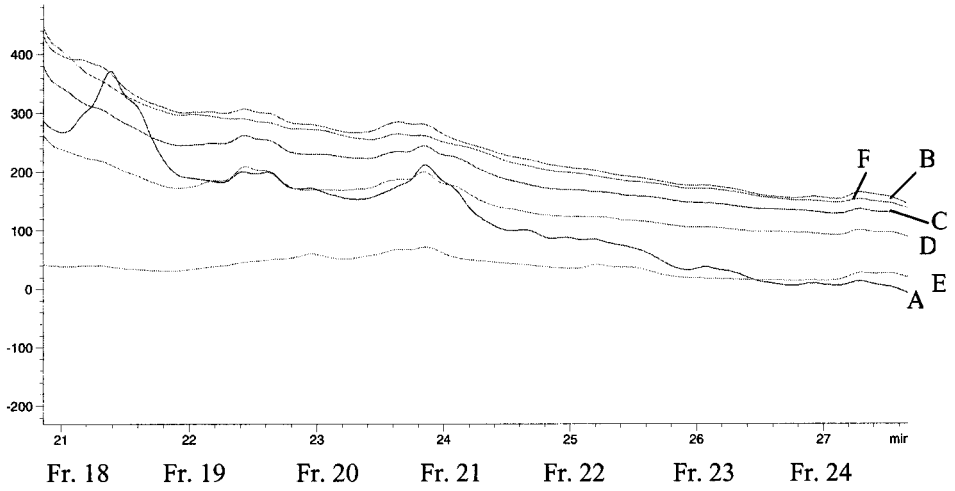

Figure 5. HPLC chromatogram of fractions 18−24 monitored with six different UV channels: A, 210 nm; B, 250 nm; C, 260 nm; D, 290 nm; E, 343 nm; F, 280 nm.

DISCUSSION

Lapcik et al. (*8*) found an isoflavone content of Czech beer in the range of 2−17 nmol/L. Only beer with a high original wort content showed a high isoflavone content. The majority of brands were in the range of 3 nmol/L of beer. According to our calibration of the in vitro assay, it is not possible to find such low isoflavone concentrations. The dose−response curves with the reference compounds are shown in Figure 2. Such a concentrated beer sample would hypothetically contain 3×10^{-7} mol of isoflavones/L. For analysis, the concentrate is diluted to a final concentration of 3×10^{-9} mol of isoflavones/L. From Table 1 it can be clearly seen that due to the low potency of isoflavones such a concentration cannot be detected.

The tested samples of lager beer did not show high transactivational activity. The hormonal activity measured as capacity to transactivate the estrogen receptor is very low.

Two other compounds from hops, xanthohumol and isoxanthohumol, have been assumed to be responsible for the estrogenic activity of beer. Recently, it has been shown that these compounds do not exert their estrogenic activity through the activation of the estrogen receptor. They influence the enzyme cytochrome P450, which is responsible for the steroid metabolism (*29*). In contrast to that is the finding of Milligan et al. (*9*). The related compound 8-prenylnaringenin is able to transactivate the estrogen receptor.

If xanthohumol and isoxanthohumol are present in

beer, we would not be able to find it by our in vitro test system. Furthermore, it has been shown by Stevens et al. (*30*) that xanthohumol and related prenylflavonoids such as desmethylxanthohumol and 3′-geranylchalconaringenin were lost during the brewing process. Besides, it has been shown that during lagering the prenylflavonoids are complexed with carbohydrates. It is known that conjugated steroids and phytoestrogens are not able to bind and transactivate the estrogen receptor.

Another agonist is suspected to be in the concentrated sample, but its transactivational capacity must be also very low. HPLC and GC-MS data showed, although these methods are very sensitive, no detectable amounts of unknown substances in the range of estrogen active fractions. From this biological action it is not likely that a human health risk arises from normal beer consumption. We consider normal beer consumption as an equivalent of an annual intake of 7 kg of ethanol. As shown by others, phytoestrogens also interfere with steroid metabolism (*31*−*37*). These effects may contribute to the reported hormonal changes observed with beer-drinking men (*10*). Further studies are required to identify the unknown compounds, and emphasis has to be put on the fate of the prenylflavonoids during processing of wort up to lagering of the beer, when complexed prenylflavonoids can split again.

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